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Laboratory rearing and vital statistics of *Diabrotica speciosa* and *Diabrotica viridula* (Coleoptera: Chrysomelidae), two species of South American pest rootworms

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■ **ABSTRACT.** Methods for rearing *Diabrotica speciosa* (Germar) and *D. viridula* (F.) in the laboratory, and their vital statistics are described. These Luperini are pests of several crops in South and Central America, and there was no previous work describing suitable methods for obtaining the numbers required for the mass production of parasitoids, assessing pathogens, or conducting host specificity studies. Handling of immatures and adults was reduced to a minimum, and materials used were inexpensive, reducing costs, labor time, and mortality related to manipulation. Larvae were reared on maize seedlings in a soil mixture, and adults were fed a dry diet. Oviposition was obtained in cups with corn seedlings wrapped in black cloth; eggs were harvested by rinsing them off the cloths with water, and sown by squirting them into maize cultures with plastic pipettes. Percentage of hatch, survival from egg to adult, and number of eggs laid per female were the highest cited yet for *D. speciosa* (*D. speciosa*: 92.6%, 25.9%, and 1164 respectively; *D. viridula*: 91%, 25%, and 1271 respectively), and similar for both species. *D. viridula* proved to be a more vigorous species than *D. speciosa*, with a longer average life span, and a slightly higher egg production, at least in present laboratory conditions.

KEY WORDS. *Diabrotica speciosa*. *Diabrotica viridula*. Laboratory rearing. Rootworms.

■ **RESUMEN. Cría en laboratorio y estadísticas vitales de *Diabrotica speciosa* y *Diabrotica viridula* (Coleoptera: Chrysomelidae), dos especies de vaquitas sudamericanas plagas de cultivos.** Se describen métodos para la cría en laboratorio de *Diabrotica speciosa* (Germar) y *Diabrotica viridula* (F.) y sus estadísticas vitales. Estos Luperini son plagas de varios cultivos en América del Sur y Central, y no había trabajos previos describiendo métodos adecuados para obtener las cantidades de adultos y larvas necesarias para crías masivas de enemigos naturales, estudios de patógenos, o pruebas de especificidad de enemigos naturales. El manipuleo de inmaduros y adultos, y su mortalidad asociada, fueron reducidos al mínimo. Los materiales utilizados fueron de bajo costo, y se redujeron las horas hombre. Las larvas se alimentaron de plántulas de maíz y los adultos de una dieta seca. Se obtuvieron puestas en recipientes con plántulas de maíz envueltas con rectángulos de tela negra humedecida; los huevos se cosecharon mediante chorros de agua de los trozos de tela y se sembraron en los cultivos de maíz mediante pipetas plásticas. El porcentaje de eclosión, supervivencia de huevo a adulto, y promedio de huevos por hembra fueron similares en ambas especies (*D. speciosa*: 92,6%, 25,9%, y 1164 respectivamente; *D. viridula*: 91%, 25% y 1271 respectivamente), y los más altos obtenidos en laboratorio hasta ahora para *D. speciosa*.

En las condiciones de laboratorio utilizadas, *D. viridula* resultó una especie más vigorosa que *D. speciosa*, con una mayor longevidad y producción de huevos.

PALABRAS CLAVE. *Diabrotica speciosa. Diabrotica viridula.* Cría en laboratorio. Chrysomelidae plagas.

INTRODUCTION

The subtribe Diabroticina has polyphagous and oligophagous species that have been recorded to feed on many food, ornamental, and wild plants. Of these, the genus *Diabrotica* Chevrolat has the greater number of pest species, including some of the most important row and orchard crops pests of the Americas. In South America, the most common and pestiferous species is *Diabrotica speciosa* (Germar). The adults are blamed for important damage in maize, as part of the kernels do not mature because they eat the silks; cucurbits, where they eat the flowers and young fruit; heavy defoliation in soya and damage to the tender parts of almost every crop (Christensen, 1943; Silva *et al.*, 1968; Link & Costa, 1978). They also may transmit bacterial wilt in cucurbits (Cabrera Walsh, unpublished data).

Although larval damage to crops has not been evaluated rigorously, there is sound evidence that they seriously damage corn, wheat, potatoes and peanuts (Sarasola *et al.*, 1980; Gassen, 1984). *Diabrotica speciosa* belongs to the *fucata* group, which contains generally multivoltine, polyphagous species, and none of them are known to have diapausing eggs, but rather overwinter as adults (Krysan & Smith, 1987). The other group within the genus, the *virgifera* group, includes the worst pests of maize in North America, based on annual losses in yields and control costs (Metcalf, 1986). This group generally shows a stricter stenophagy, especially of larval hosts, diapausing eggs, and univoltinism (Krysan & Smith, 1987). The sole South American species of this group that may arguably be considered a pest, is *Diabrotica viridula* (F.), reported to cause significant damage on maize (Harries, 1975; Olalquiaga, 1980), and to transmit maize chlorotic mottle virus in Peru (Reyes & Castillo, 1988).

Studies on these insects have led to the development of several laboratory rearing procedures. Most of these methods involve rearing larvae on a live host, typically corn seedlings, and feeding the adults meridic diets, fresh food (bean leaves, squash leaves and fruit, corn seedlings), or both (Mendoza & Peters, 1963; Howe & George, 1966; Chalfant & Mitchell, 1968; Cuthbert *et al.*, 1968; Skelton & Hunter, 1970; Branson *et al.*, 1975; Jackson, 1986; Schalk, 1986; Campbell & Jackson, 1987; Branson & Jackson, 1988; Branson *et al.*, 1988). Several attempts to rear immature stages of *Diabrotica* on artificial diets have been successful (Sutter *et al.*, 1971; Rose & McCabe, 1973; Marrone *et al.*, 1985; Avila *et al.*, 2000). However, they have proven less effective and more labor intensive than the live hosts methods, so Diabroticites are, but exceptionally, at present reared on live hosts.

The objectives of this work are to report the results of the methods developed at the ARS-USDA South American Biological Control Laboratory (SABCL), Hurlingham, Argentina, for rearing *D. speciosa* and *D. viridula*, and their vital statistics, with notes on their development and biology.

MATERIAL AND METHODS

Field Collections. The founding beetles of the *D. speciosa* and *D. viridula* colonies were adults collected in the field throughout northeastern and central Argentina. Eggs were harvested from the field collected adults, reared in the laboratory, and the adults mixed with the laboratory colony, to maintain a diverse gene pool.

The beetles were usually collected off foliage by sweeping. Corn silks or flower heads were knocked over the edge of a plastic funnel attached to a plastic container. In delicate crops, such as lettuce, where sweeping would cause extensive damage, they were collected with hand aspirators. Battery–operated aspirators and a D–vac also have been tried, but they have not proven useful, because they clog with leaves and petals easily. Together with these methods, polyester cloths sprayed with alcoholic *Cayaponia spp.* root or berry extracts were deployed at every collection site, to attract beetles to them while the workers collected on the plants (Cabrera Walsh, unpublished

data). When collecting was complete, the cloths were shaken into a sweeping net, which was emptied of its contents of beetles into a cage, or separated by species on the spot with aspirators.

Laboratory Handling. Two sizes of cylindrical cages with sleeves (1.5 and 19 liters) were used for rearing adults and collecting their parasitoids. The small containers can hold ca. 150 beetles, while the large ones up to 1500 beetles. Both had a size 12 plastic mesh attached to the bottom through which emerging parasitoids fell onto a moist sand bed. Adults were fed a diet based on Campbell & Jackson (1987), to which antimicrobials were added (Table 1). Water was provided from 45 ml plastic cups with cotton wool wicks through the lids.

Rearing. Oviposition was obtained in 42-ml plastic cups with moist squares of folded black cotton/polyester cloth, with maize seedlings rooted amidst the folds. Females laid their eggs in the folds. The seedlings were obtained by incubating 40-50 kernels at 25 °C in 0.5-liter plastic containers with 3 cm of moist oven-sterilized sand. When the seedlings reached 3-7 cm in height, the sand was washed away, and the seedling roots (5-7 per cup) wrapped in the cloths, pressed into the cups, and moistened thoroughly. Although Diabroticites oviposit on several moist substrates, such as sand, soil, cotton wool, peat moss, and paper, the presence of a fresh food stuff, and especially the larval host, greatly stimulates oviposition (Branson *et al.*, 1975; Cabrera Walsh, unpublished data). The eggs were rinsed off the seedlings and cloths into a beaker with water. The contents of the beaker were strained to retain root and foliage debris. The eggs were then allowed to settle and the excess water was eliminated together with floating debris. This process was repeated to eliminate as much foreign matter as possible, so the eggs were obtained relatively free of foreign matter. This cleaning process was important to obtain a close egg estimation. The number of eggs sown in each container was estimated by using a 1-ml glass pipette with the tip cut off to where the tube starts tapering, and a piece of nylon mesh stuck to the tip. The eggs are fed down the mouth, suspended in water, with a pipette with a bulb. Once the required volume of eggs was reached, they were rinsed out the mouth. The measuring pipette is marked every 100 eggs, based on the average volume of five previously counted samples of 1- to 3-d-old eggs. The samples were allowed to settle for some 30 s, and tapped to eliminate air bubbles. The same procedure was used for *D. viridula*, including collection methods, laboratory handling, rearing, and egg processing.

Table 1. Diets used for *Diabrotica speciosa*, and *D. viridula* (for 1Kg of diet)

Ingredients	Amounts
Soy flour	54.5 g
Wheat germ	54.5 g
Casein	70 g
Alphacel	82 g
Sucrose	145 g
Brewers yeast	22 g
Glicerine	60 g
Wesson salts mix	5.5 g
Vanderzant vitamins mix	5.5 g
Cholesterol	1.0 g
Methylparaben	0.65 g[a]
Sorbic acid	0.92 g[a]
Acetic acid	2.7 ml
Distilled water	500 ml

[a] dissolved together in 8ml of ethanol.

Blend the mixture with part of the water after each ingredient is added. Squeeze 5-mm thick, 2-cm wide rows on successive layers of aluminum foil and freeze. Thaw in microwave and spread on shallow lid. Renew every week-10 d or when consumed.

The eggs collected from the oviposition cups were either sown on the spot, or chilled at 5 ± 1 °C in petri dishes with moist blotting paper until needed. Tests on eclosion percentage were performed by incubating five groups of ca. 100 eggs of each species at 25 °C in petri dishes with moist blotting paper. The proportion of eggs hatched in each subsample was averaged for each species. Also, four samples of ca. 2000 eggs from each species were chilled at 5 ± 1 °C in petri dishes with moist blotting paper, and subsamples of ca. 100 eggs were washed off the paper every 3-4 d, and incubated in petri dishes with moist blotting paper at 25 °C. The proportion of eggs hatched was recorded and averaged to measure the survival of chilled eggs.

The larval rearing method was based on the techniques used in most mass rearing procedures used for the North American *Diabrotica* species. The larvae were incubated on corn seedlings in a

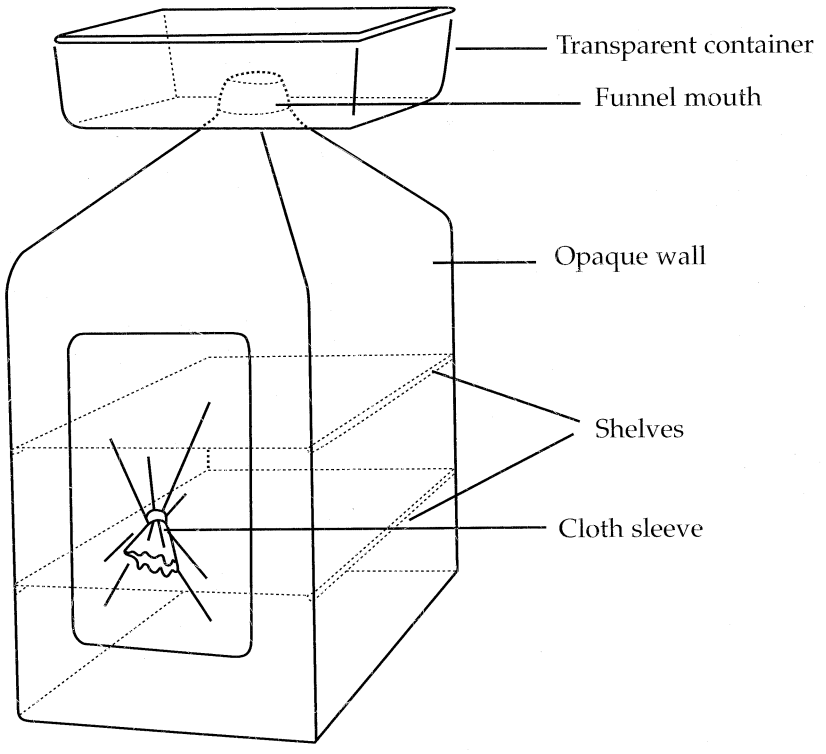

Fig. 1. Emergence cage.

soil mixture (1:1 v/v sandy loam: peat moss, 40% w/w water contents, autoclaved 20 minutes). Around 200-600 eggs were spread with a pipette on a 3-cm layer of soil mixture in 0,5-liter round squat plastic containers, with about 60 maize kernels that had been previously soaked in 3% detergent for 3 h, to reduce fungal growth, and water for another 20 h, to obtain uniform and quick sprouting. The container was covered with a plastic lid that had a 2-cm opening covered with a fine mesh. After 8-10 d (four to seven days after eclosion) at 25-28 °C, the contents were transferred into a 2-liter rectangular squat plastic container with a similar opening in the lid, and around 60 seedlings in 3 cm of soil mixture. The seedlings were grown around the sides so as to allowed the transference of the whole contents of the smaller round containers to the center of the renewal culture, without injuring them. The seedlings grew vigorously; it was never necessary to add water to keep suitable moisture, and the coarse structure of the soil mixture allowed free movement of the larvae. Finally, when the first adults emerged, the containers were transferred without the lid to an emergence cage (Fig. 1) consisting of a steel frame with plywood walls and a cloth sleeve. The top of the cage tapers to a funnel that is pressed into a 5-liter transparent container. The cage has three sets of rails for three shelves on which the rearing containers were piled. When the young beetles emerged, they would climb into the transparent container, which is the only light source of the cage, thus efficiently collecting all the adults as they emerge. Young beetles were provided slices of squash in the emergence container to increase survival.

A laboratory test on reproduction was conducted to obtain the mean longevity, mean oviposition per female, survival curves (l_x as a fraction of an initial population of one) (Southwood, 1978), and timing of mating from emergence. Small groups of beetles of both species (8-5 males and females), and single females with five males, were incubated from day one after emergence in 2-liter cages (six repetitions of small groups and six of single females) at 25 °C. The cages were provided with dry diet, water, and oviposition cups with maize seedlings. Mating, oviposition, and mortality were recorded every 3-5 d until death of the colonies.

Developmental tests at 32, 28, 25, 20, 15, and

13 °C (± 1 °C for all), with different initial egg numbers (100, 200, 300, 400, 500, 600, 800, and 1000-1200 eggs per container) were conducted to obtain immature stage duration data, and determine the most efficient incubation conditions. Also, different periods elapsed in the initial rearing containers before transference to the renewal cultures (5-7 d, 9-11 d, and 13 + d) were recorded. The results of rearing the larvae at different temperatures, transference period, and initial egg densities were compared with an ANOVA, and post hoc multiple comparisons (SYSTAT, 1996).

RESULTS

Field Collections. A total of 79000 *D. speciosa* were collected between April 1994 and April 2001. Roughly 50% of beetles were collected from cucurbits, 20% from corn, 10% from sunflower, 10% with baits (in use since May 1999), and the rest from the alternative crops mentioned above.

Except for 12 specimens collected in Salta province, Argentina, in 1994, *D. viridula* has only been found regularly in northern Argentina and neighboring areas since the beginning of 1999. A total of 2960 specimens were collected, roughly 45% on corn, 40% on sunflower, and 15% on cucurbits.

Laboratory Colonies and Reproduction. Eggs of *D. speciosa* refrigerated at 5 ± 1°C were viable for a maximum of 60 d, with a steady decline in viability from the 10th d, but high eclosion rates lasted ca. 20 d. Initial eclosion success at 25 ± 2 °C averaged 92.6%, and took 8 ± 1 d. *Diabrotica viridula* eggs hatched in 9 d (mean = 91%), and chilled eggs were viable for a maximum of 40 d (Fig. 2).

These species have three instars, which are easily differentiated with the naked eye by the size of the head capsule (*D. speciosa* head capsule width of instar I, 0.23 mm; instar II, 0.36 mm; instar III, 0.56 mm; and *D. viridula* head capsule width of instar I, 0.21 mm; instar II, 0.34 mm; instar III, 0.52 mm). At 25 °C the larval stage of *D. speciosa* lasted around 17 d, including an inactive prepupal period of 2-3 d. The pupal stage lasted 6 d, and was followed by a period of 3 to 5 d during which tenerals remained in the pupal cell, presumably for the cuticle to tan. The time for peak emergence from egg to adult for *D. speciosa*

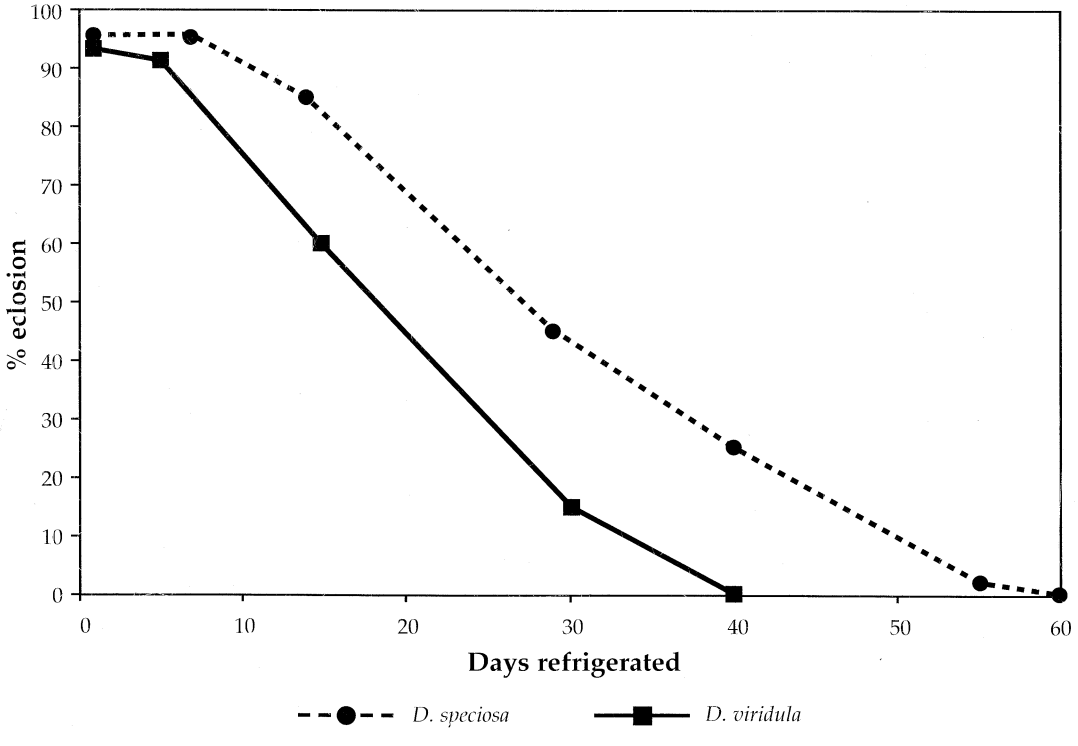

Fig. 2. Percent eclosion of eggs chilled at 5 (± 1) °C of *D. speciosa*, and *D. viridula*.

Table 2. Rearing statistics for *Diabrotica speciosa* in the laboratory.

Response to egg density		Response to incubation temperature[a]		Response to time in first culture[a]	
eggs/container	% adult emergence	temp (°C)	% adult emergence	transf. to new culture (days)	% adult emergence
100-200	32 a	20-22	20	5-7	24
300	18 ab	25	19.8	9-11	18
400	18 ab	28	15.9	13 +	18
500	22 ab	30-32	12.4		
600	14 b				
700-800	11 b				
900 +	3 c				

[a] Data from densities with non significant differences (300-800 eggs/container).
Cells with the same letter indicate non significant differences.

Table 3. Rearing statistics for *Diabrotica viridula* in the laboratory.

Response to egg density		Response to incubation temperature[a]		Response to time in first culture[a]	
eggs/container	% adult emergence	temp (°C)	% adult emergence	transf. to new culture (days)	% adult emergence
100-200	25 a	20-22	17.8	5-7	16.7
300-400	19 a	25	18.8	9-11	18.7
500-600	15 a	28	16.4	13 +	18
800 +	5.7 b				

[a] Data from densities with non significant differences (100-600 eggs/container).
Cells with the same letter indicate non significant differences.

(the day when the median of the beetles emerged) was 39, 32, 30, 28, and 33 d at 20, 25, 28, 30 and 32 °C respectively. The emergence period (lapse since the first to the last adults emerge) extends for 11, 10, 8, 7, and 13 d, respectively. A nonsignificant higher larval survival was detected under 28 °C, so the larvae were reared at 25 °C (Table 2). At 15 °C, few beetles completed development, and only after ca. 65 d. At 13 °C, no larvae hatched.

The larval stage of *D. viridula* lasted 19 d, plus a 7-d pupal stage, totaling an average development time from egg to adult at 25 °C of 34 d. Emergence of *D. viridula* at 25 °C spanned 9 d, with peak at day 36. At 28 °C, developmental time was reduced to 31 d, and emergence of every adult was observed in the course of five days. Young beetles of both species were yellowish or pale brown, and turned green with bright yellow spots in 3 d if fresh food was provided. Beetles fed on artificial diets or pollen exclusively never turned green.

Laboratory–reared beetles went through a period of about 2 wk during which they were soft and delicate, and mortality fluctuated between 15 and 24%, after which the remaining beetles lived on for up to 100 d in the case of *D. speciosa* ($\bar{X}$= 55.7 d, SD = 29.1), and 130 d for *D. viridula* ($\bar{X}$ = 83.4 d, SD = 39.4).

The number of eggs surviving to adulthood varied significantly with the number of eggs sown. The optimal number of eggs per container for *D. speciosa,* as regards survival, was 100-500, with 25.9% of the eggs reaching adulthood (F = 5.08; df = 5. 59; P = 0.001). The following group, 600-800 eggs per container, showed a significantly lower survival than the 100-200 group (14%), which was, however, not significantly lower than the 300-500 group. However, considering the space and materials requirements (specially sterile soil mixture) of this rearing method, around 500 to 800 eggs per container is considered to be the optimal num-

ber. Also, there was a non-significant higher adult emergence when the first culture was transferred to new seedlings at ≤7 d (Table 2).

For *D. viridula*, the same densities of eggs per container were compared. The ANOVA yielded significant differences between groups ($F = 4.138$; df = 3. 21; $P = 0.019$), but the pairwise comparisons only showed differences between the 800 + group and the 100-600 group. So the optimal number of eggs per container is considered at 600. No significant differences were detected between times elapsed from the sowing of the eggs and transference to renewal cultures (five to 14 d), or rearing between 20 and 28°C (Table 3).

Mating for both species was observed between days 4 and 6 after emergence, and some females were observed mating again at ca. day 35. Each female *D. speciosa* laid an average of 1164 eggs (SD = 324.03) during her lifetime, starting on day 8 and extending for a maximum of 85 d. Peak oviposition was observed between days 23 and 49, although the number of eggs recovered every 3 d varied drastically during this period (Fig. 3).

Diabrotica viridula females laid an average total of 1271 eggs (SD = 69.4), starting on day 5 and extending for a maximum of 100 d. Peak oviposition, but with a marked cleft between days 30 and 60, was observed between days 13 and 75 (Fig. 4). Together with a slightly higher oviposition per female, under the same laboratory conditions *D. viridula* lived longer, showed a longer oviposition period, and a lower initial mortality compared with *D. speciosa* (Fig. 4).

Although the proportion of mating females was not assessed directly, virgin females kept for life in cages for feeding tests (Cabrera Walsh, unpublished data) did not lay any eggs. Based on the assumption that every female that would lay eggs was, thus, mated, the percentage of mated females was 93.2%. Females that did not lay eggs were removed from the statistics.

DISCUSSION

Generally speaking, *D. viridula* is a more vigorous species than *D. speciosa*, having a longer average life span, and a slightly higher fecundity, at least under present laboratory conditions (Figs. 3 and 4). However, it shows more constraints in

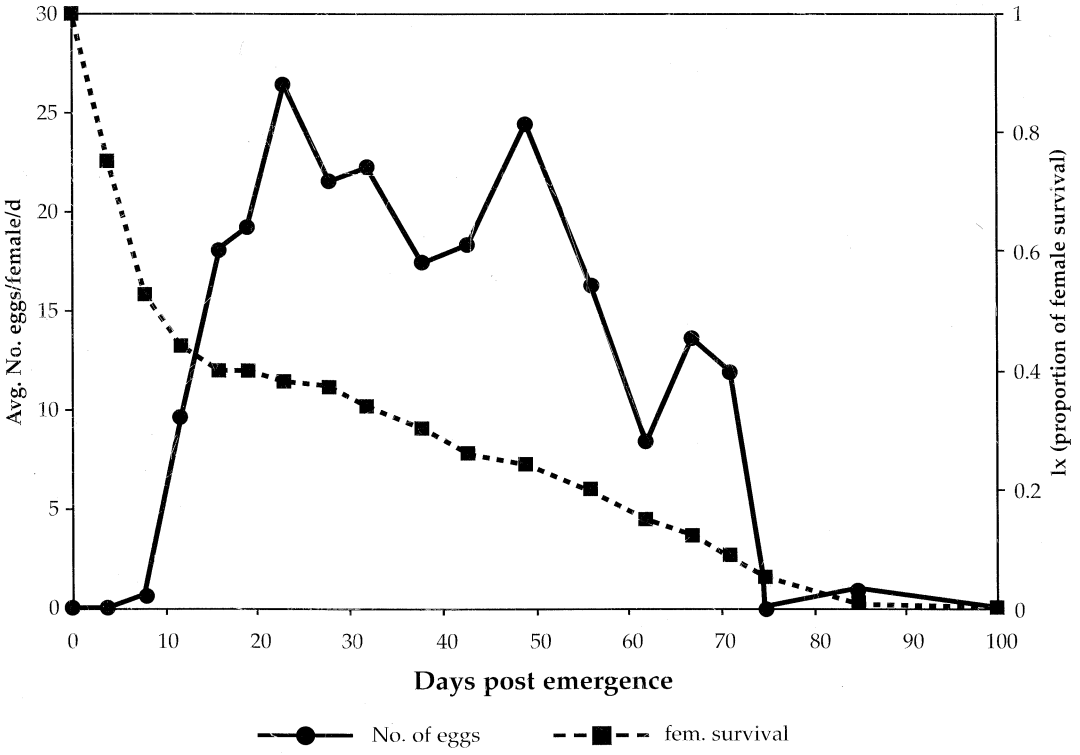

Fig. 3. Average daily oviposition per single *D. speciosa* female, and survival curve (lx) relative to an initial population of 1 female.

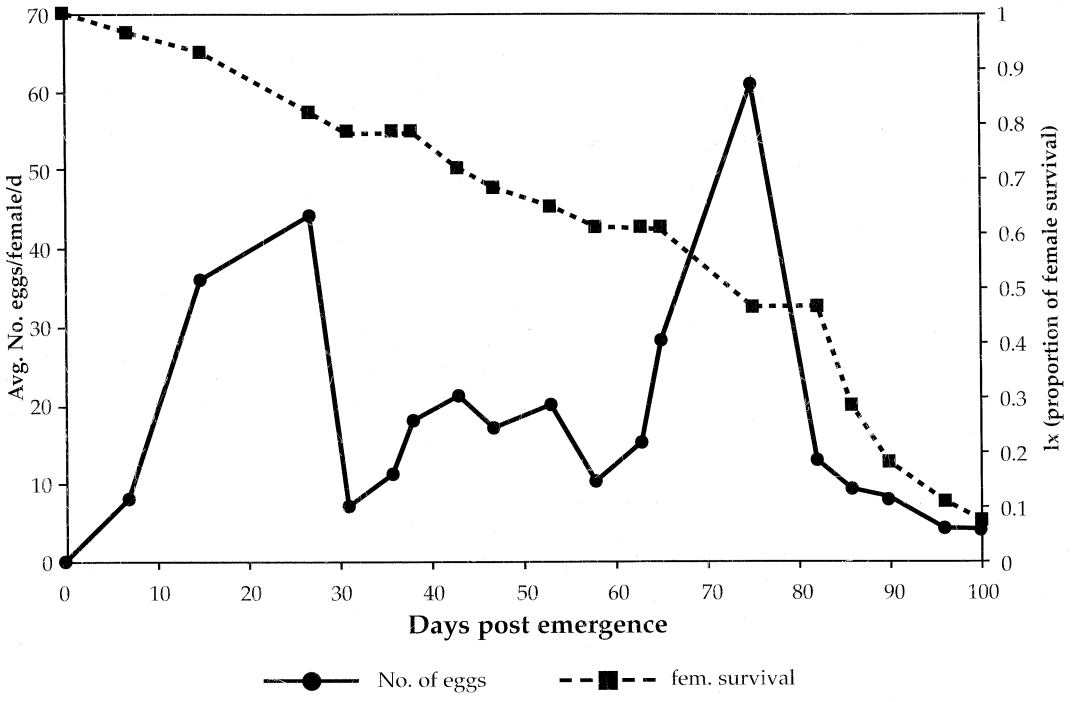

Fig. 4. Average daily oviposition per single *D. viridula* female, and survival curve (lx) relative to an initial population of 1 female.

egg laying after exposure to low temperatures, and the eggs show a lower capacity for being dormant under low temperatures (Fig. 2).

The standardized methods used currently at the SABCL allow permanent colonies of *D. speciosa* and *D. viridula*, which provide a stock of adults and larvae for breeding, host range experiments with the parasitoids in culture, and pathogenicity experiments with pathogens isolated from field specimens. Provided suitable facilities, the method is adaptable to virtually any production requirement, both small numbers used for laboratory tests, or mass production.

In previous studies on *D. speciosa* rearing (Martinez de Carvalho & Hohmann, 1983; Pecchioni, 1988; Laguzzi & Novara, inéd; Silva–Werneck *et al.*, 1995), the developmental statistics reported were notably different between the different works. However, in this study such variables as eggs per female, longevity, percent hatching, percent adults emerged, preoviposition period, and developmental time were consistently improved. The reasons for this are possibly less manipulation of fragile stages as the eggs and pupae, and better nourishment. The diets described herein are more similar to pollen, *Diabrotica* adults first choice in the field. Whereas other methods involved mainly feeding the adults fresh food (leaves and seedlings), with lower food value. This method is also less labor intensive and more economical. Also, even though small numbers of eggs (<200) per rearing container provided better egg to adult survival, considering eggs are rarely a limiting factor, 500 to 800 eggs per container allowed good survival plus a significant economy of space and materials.

The statistics obtained in this work can be used for life and fertility tables, that could describe theoretical reproductive traits of the species in the field. However, caution must be taken when extending these statistics to a natural situation, although they may be of use to obtain conservative estimations of maximum population growth rates.

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